PD-L1 expression is an independent predictor of favorable outcome in patients with localized esophageal adenocarcinoma

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Abstract: Background. The outcome of patients with adenocarcinoma of the esophagogastric junction (AEG) remains poor. The programmed cell-death-protein-1 (PD-1), a co-inhibitory receptor primarily expressed by T-cells, represents a potential new therapeutic target. PD-1, PD-1 ligand 1 (PD-L1), and PD-L2 expression have all been described as prognostic factors in a variety of cancers. Their expression patterns in AEG, however, are poorly understood. We analyzed PD-L1, PD-L2 and PD-1 expression by tumor-infiltrating lymphocytes (TILs) and cancer-cells in tumor-biospecimens in AEG-patients. Methods. 168 patients who underwent esophagectomy because of AEG between 1992-2011 were included in this study. PD-L1, PD-L2 and PD-1 expression were evaluated by immunohistochemistry and correlated with various clinicopathological parameters, disease-free survival (DFS) and long-term overall survival (OS). Results. PD-L1 expression by cancer-cells (cancer-cell-PD-L1+) was found in 43.5% of patients whereas PD-L1 expression by TILs (TILs-PD-L1+) was observed in 69%. PD-L2 expression by cancer-cells and TILs was only found in 3.5% and 1.8%, respectively. Additionally, 77.4% of tumors contained PD-1+-cancer-cells and 81% PD-1+-TILs. Patients with increased expression of PD-1 by cancer-cells and TILs showed significantly reduced OS and DFS, as determined by univariate, but not multivariate analysis. Expression of PD-L1 by cancer-cells was found to be an independent predictor for improved DFS ($p = 0.038$) and OS ($p = 0.042$) in multivariate analysis. Conclusions. Cancer cells and TILs displayed PD-L1 expression in around 50% and PD-1 expression in around 80% of tumor-biospecimens obtained from AEG patients. Expression of PD-L1 is an independent predictor of favorable outcome in AEG, whereas PD-1 expression is associated with worse outcome and advanced tumor stage.

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Abstract

Background. The outcome of patients with adenocarcinoma of the esophagogastric junction (AEG) remains poor. The programmed cell-death-protein-1 (PD-1), a co-inhibitory receptor primarily expressed by T-cells, represents a potential new therapeutic target. PD-1, PD-1 ligand 1 (PD-L1), and PD-L2 expression have all been described as prognostic factors in a variety of cancers. Their expression patterns in AEG, however, are poorly understood. We analyzed PD-L1, PD-L2 and PD-1 expression by tumor-infiltrating lymphocytes (TILs) and cancer-cells in tumor-biospecimens in AEG-patients. **Methods.** 168 patients who underwent esophagectomy because of AEG between 1992-2011 were included in this study. PD-L1, PD-L2 and PD-1 expression were evaluated by immunohistochemistry and correlated with various clinicopathological parameters, disease-free survival (DFS) and long-term overall survival (OS). **Results.** PD-L1 expression by cancer-cells (cancer-cell-PD-L1^{+}) was found in 43.5% of patients whereas PD-L1 expression by TILs (TILs-PD-L1^{+}) was observed in 69%. PD-L2 expression by cancer-cells and TILs was only found in 3.5% and 1.8%, respectively. Additionally, 77.4% of tumors contained PD-1^{+}-cancer-cells and 81% PD-1^{+}-TILs. Patients with increased expression of PD-1 by cancer-cells and TILs showed significantly reduced OS and DFS, as determined by univariate, but not multivariate analysis. Expression of PD-L1 by cancer-cells was found to be an independent predictor for improved DFS (p=0.038) and OS (p=0.042) in multivariate analysis. **Conclusions.** Cancer cells and TILs displayed PD-L1 expression in around 50% and PD-1 expression in around 80% of tumor-biospecimens obtained from AEG patients. Expression of PD-L1 is an independent predictor of favorable outcome in AEG, whereas PD-1 expression is associated with worse outcome and advanced tumor stage.

Key words

PD-1, PD-L1, PD-L2, esophageal adenocarcinoma, esophageal carcinoma

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Disclosure of interest

The authors report no conflict of interest

Abbreviations

AEG – adenocarcinoma of the esophagogastric junction

CI – confidence interval

DFS – disease free survival

ESCC – esophageal squamous cell carcinoma

HR – hazard ratio

ICH – Immunohistochemistry

IQR – interquartile range

NSCLC – non small cell lung cancer

OS – overall survival

PD-1 – programmed cell death 1 receptor

PD-L1 – programmed cell death 1 receptor ligand 1

PD-L2 – programmed cell death 1 receptor ligand 2

RT – room temperature

TILs – tumor infiltrating lymphocytes

TME – tumor microenvironment

Introduction

The prevalence of esophageal cancer is increasing, and it currently represents the eighth most common cancer and sixth most common cause of cancer-related death worldwide.[1,2] The 5-year overall survival (OS) rates for patients with esophageal carcinoma range from 10-15%, with only moderate improvement over the past years. [3] Eesophageal carcinoma treatment includes surgical resection, as well as cytotoxic chemotherapy, radiotherapy, or targeted treatment modalities (e.g. monoclonal antibody directed against HER2). [3,4] Therapeutic antibodies targeting the programmed cell death 1 (PD-1) pathway have made substantial inroads in the treatment of several advanced cancers of various etiology, and may thus also represent an additional therapeutic option in AEG patients. [5,6] PD-1 is an immune checkpoint receptor and prominent mediator of tumor immune evasion, that is primarily up-regulated by activated T cells, including TILs. Multiple tumor entities have been studied for their expression of the PD-1 ligands, PD-L1 and PD-L2. [7] Increased PD-1 ligand expression in the tumor microenvironment (TME) was reported to be associated with poor prognosis in several tumors (e.g. renal cancer, breast cancer, hepatocellular carcinoma, and colorectal cancer), consistent with the established role of the PD-1:PD-1 ligand axis in tumor immune evasion. [8-14] Accordingly, both PD-1 and PD-L1 blocking antibodies can activate tumor-specific immune response by overcoming PD-1 pathway-mediated immune escape. Indeed, excellent results have been reported in multiple large-scale clinical trials involving PD-1/PD-L1 blocking antibodies for the treatment of advanced solid tumors and hematologic malignancies.[15,16,17] To date, several studies have investigated the expression pattern of PD-1 and its ligands in esophageal squamous cell carcinoma (ESCC). [18-22] However, only limited data is available on the PD-1 pathway in esophageal adenocarcinoma. In this study, we aimed to further elucidate the role of PD-1, PD-L1 and PD-L2 expression in esophageal

adenocarcinoma. Specifically, we systematically examined the prognostic impact of PD-L1 and PD-L2 expression by cancer cells and by TILs on DFS and OS in a large cohort of patients undergoing esophagectomy for esophageal adenocarcinoma.

Methods

Study population

Patients undergoing esophageal resection for adenocarcinoma of the esophagogastric junction (AEG) between the years 1992 and 2011 (n=168) at the Department of Surgery, Medical University of Vienna, were included. Data were collected from the institutional database and individual patient charts were reviewed. According to the institutional policy, post-surgical follow-up was conducted in three-months intervals during the first year after esophagectomy, in six-month intervals during the following three years, and yearly thereafter. Histological analyses were performed in a large collection of archived tissue samples of resected tumors. Approval for the study has been obtained by the local ethical committee of the Medical University of Vienna (# 1056/2016).

Immunohistochemistry (IHC)

Immunohistochemistry (IHC) was performed on 3-5 µm thick paraffin sections as previously described.[23] The sections were deparaffinized and rehydrated in graded series: X-TRA-Solv 8 (Medite, # 41-5212-00) - 15 min at 68°C; Xylol – 5 min room temperature (RT), 100% EtOH - 5 min RT; 96% EtOH - 5 min RT; 80% EtOH - 5 min RT; distilled water - 2 min RT. For antigen retrieval, the slides were heated in a Dako Cytomation Pascal Pressure Cooker (115°C) and after that, endogenous peroxidase activity was blocked using 3% hydrogen peroxide in distilled water (10 min). Normal goat serum was used to block non-specific epitopes (30 min) and after that the sections were incubated with the following primary antibodies: mouse anti-human PD1 (R&D systems, # AF 1086, dilution 1:20), PD-L1 (Cell signaling, clone: E1L3N, dilution: 1:25), PD-L2 (Cell signaling, clone: D7U8C, dilution: 1:25) as well as the corresponding

biotinylated anti-goat IgG secondary antibody (1:100 dilution, 30 min). Following manufacturer's protocol (Dako), visualization was achieved via application of streptavidin conjugated to alkaline phosphatase. Additional Mayer's hematoxylin staining was applied in order to depict the cell nuclei.

The tumor samples were investigated blinded to patients' clinical data on one slide per patient. On lower magnification (40x), the pattern and distribution of PD-L1, PD-L2 and PD-1 expression over the whole area of the tumor was assessed. In case of heterogeneity of the expression patterns within individual tumor lesions, we selected four visual fields representing all patterns of levels of PD-L1, PD-L2 and PD-1 expression present in the studied lesion (x400). PD-L1, PD-L2 and PD1 expression was analyzed separately for cancer cells and TILs. The percentage of cancer cells and lymphocytes showing immuno-reactivity to PD-L1, PD-L2 and PD-1 was rated (positive staining, 0-100%) and classified: 0: no positive cells, 1+: 1-25% of cells, 2+: 26-50% of cells, 3+: 51-75% of cells and 4+: 76-100% of cells. Histological analyses were performed by three pathologists that were blinded to the clinical characteristics of each patient. The slides were independently graded and if the rating differed, the slides were re-discussed using a multi-head microscope and a consensus was found. For some analysis, expression was divided in PD-L1 negative ($PD\text{-}L1^{-}$) and PD-L1 positive ($PD1^{+}$).

Statistical analysis

Overall survival (OS) was defined as time between primary surgery and the patient's death. Disease free survival (DFS) was defined as time from primary surgery until first evidence of disease-progression. Patients without complete resection (n=23) were excluded form the analyses of OS as well as DFS.

Median follow up and interquartile range (IQR) of median follow up was estimated by reverse Kaplan-Meier method. Kaplan-Meier curves were plotted to investigate differences in OS and DFS between PD-L1 expression patterns of cancer cells and TILs, respectively.

Differences in clinicopathological parameter distributions between patients with PD-L1^{-} and PD-L1^{+} expression were assessed by Fisher's exact test and by its extension for >2x2 tables in case of parameters with more than 2 levels. Clinicopathological parameters included categorical variables only.

Univariate Cox proportional hazards models were carried out to estimate the effect of PD-L1 and PD-1 expression of cancer cells and of TILs as well as other clinicopathological parameters on OS and DFS, separately. Stepwise regression analysis was applied to select the set of predictors that best predict OS and DFS, respectively, in the setting of a multivariate Cox proportional hazard model. PD-1 expression by tumor cells and TILs were kept in the model for comparison with previous results. Proportional hazard assumptions were assessed visually and tested using diagnostics based on weighted residuals. All tests were two-sided and p-values less than 0.05 were considered statistically significant. All statistical analyses were performed with the statistical software R version 3.33 (R Development Core Team, 2017). [24]

Results

Patients' characteristics

In total, 168 patients who had undergone esophagectomy for esophageal adenocarcinoma between 1992 and 2011 were included in this study. The mean age at the time of surgery was 65 years (range 35-88 years, ±10.4 years) and the ratio of female to male patients was 31:137. The median follow-up time for OS was 66 months (IQR 28-96 months). OS rates of patients with complete resection (n=145) at one, 5 and 10 years were 78.6% (107 patients at risk), 49.9% (54 patients at risk) and 37.3% (12 patients at risk), respectively. The median follow up for DFS reached 61 months (IQR 28-96 months). DFS rates at one, 5 and 10 years were calculated as 72.6% (79 patients at risk), 48.7% (31 patients at risk) and 39.7% (6 patients at risk), respectively. Neoadjuvant therapy was applied in 63 patients (37.5%) (neoadjuvant chemotherapy (n=59) and neoadjuvant radiochemotherapy (n=4)). Tables 1 and 2 summarize clinical and histopathological data.

Expression of PD-L1 in cancer cells and TILs

PD-L1 expression was determined by IHC and quantitated separately for cancer cells and TILs. PD-L1 expression by cancer cells was detected in 73/168 patients (43.5%), with PD-L1$^+$ cancer cell frequencies distributed as follows: 0 (0%): n=95 (56.5%), 1+ (1-25%): n=51 (30.4%), 2+ (26-50%): n=12 (7.1%), 3+ (51-75%): n=8 (4.8%), 4+ (76-100%): n=2 (1.2%) (Table 1). Tumor biospecimens from 116/168 patients (69%) contained PD-L1$^+$ TILs, categorized as: 0 (0%): n=52 (31%), 1+ (1-25%): n=77 (45.8%), 2+ (26-50%): n=29 (17.3%), 3+ (51-75%): n=10 (6%) and 4+ (76-100%): n=0 (0%) (Table 2). Representative images for (a) negative (0) PD-L1 staining on cancer cells, (b) 2+ and (c) 3+ positive PD-L1 staining on cancer cells as well as (d) negative

(0) PD-L1 staining on TILs (e), 2+, and (f) 4+ positive PD-L1 staining by TILs are depicted in Figure 1.

Expression of PD-L2 by cancer cells and by TILs

PD-L2 expression was evaluated separately for cancer cells and TILs by IHC staining. Only 6 patients with esophageal adenocarcinoma (3.6%) showed positive PD-L2 staining by cancer cells (1+ (1-25%): n=5 (3%) and 2+ (26-50%): n=1 (0.6%)). Two of these patients also harbored PD-L1$^+$ cancer cells, and 5 PD-L1$^+$-TILs in the respective tumor biospecimens. Furthermore, only 3 tumors (1.8%) were found to contain PD-L2$^+$ TILs, all of which were classified as 1+ (1-25%) and also positive for PD-L1 on both cancer cells and TILs.

Comparison of tumors with or without PD-L1$^+$ cancer cells

Patients were separated into 2 groups: those containing PD-L1$^+$ cancer cells (n=73) and those negative for cancer cell-PD-L1 (n=95); clinicopathological findings were evaluated and compared between both groups (Table 1). Tumors with PD-L1$^+$ cancer cells were more likely found during earlier tumor stages compared to patients with cancer cell-PD-L1$^-$ tumors (p=0.045; pT1a+pT1b: n=21/73 (28.8%) vs. 12/95 (12.6%), pT2 14/73 (19.2%) vs. 35/95 (36.8%), respectively, Table 1). However, a similar number of patients were classified as pT3 when comparing tumors with and without PD-L1$^+$-cancer cells (33/73 (45.2%) vs. 44/95 (46.3%), Table 1). There were no significant differences between the two groups in lymph node status (p=0.520), histologic grading (p=0.584), or anatomical AEG location (p=0.072), as determined by the Siewert classification (Table 1), and a similar number of patients received neoadjuvant therapy in both groups (26/73 (35.6%) vs. 37/95 (39%), p=0.748, Table 1). PD-1 receptor expression patterns, on the other hand, significantly differed between

PD-L1^{+} and PD-L1^{-}-cancer cell groups (p=0.016). For instance, from 38/168 (22.6%) patients with tumors negative for cancer cell-PD-1, 63.2% (24/38) nevertheless harbored PD-L1^{+} cancer cells. Similarly, 81 of the 130 patients demonstrating tumorcell PD-1 expression (62.3%) did not contain PD-L1^{+} cancer cells (Table 1).

Comparison of tumors with or without PD-L1^{+} TILs

Tumors containing PD-L1^{+}-TILs (n=116) were compared to those without detectable TIL-PD-L1 expression (n=52) in terms of clinicopathological findings (Table 2). Both groups were similarly distributed with respect to tumor stage (p=0.128), lymph node status (p=0.396), histologic grading (p=1), or Siewert classification (p=0.252) (Table 2). The number of patients who received neoadjuvant therapy was also similar in both groups (45/116 (38.8%) vs. 18/52 (34.6%), p=0.730, Table 2). However, TIL-PD-1 expression patterns were significantly different in both groups (p=0.034, Table 2). Tumors lacking PD-1^{+} TILs (32/168, 19.1%) were PD-L1^{+} in 75% (24/32) and PD-L1^{-} in 25% (8/32) of cases (Table 2). Conversely, 67.7% (92/136) of tumors containing PD-1^{+} TILs (136/169, 80.9%) also harbored PD-L1^{+} TILs (Table 2).

Correlation of PD-L1 expression by cancer cells and TILs with OS and DFS

To evaluate the impact of PD-L1^{+} cancer cells and TILs on OS and DFS, PD-L1 expression patterns were grouped into three subcategories: 0 (0%, PD-L1^{-}), 1 (1-25%, PD-L1^{+}) and 2+ (26-100%, PD-L1^{++}), respectively. DFS increased significantly with an increase in PD-L1 expression by cancer cells (DFS: p=0.027, one-, 3- and 5-year DFS rates: cancer cell-PD-L1^{++} 89%, 77%, 77%; cancer cell-PD-L1^{+} 72%, 49%, 37%; cancer cell-PD-L1^{-} 59%, 39%, 31%, respectively, Figure 2a). Additionally, OS was significantly higher in patients with higher cancer cell-PD-L1 expression (OS: p=0.045, one-, 3- and 5-year OS rates: cancer cell-PD-L1^{++} 95%, 76%, 76%; cancer cell-PD-

L1^{+} 87%, 55%, 39%; cancer cell-PD-L1^{-} 68%, 45%, 36%, respectively, Figure 2b). The comparison of DFS and OS between PD-L1^{++}, PD-L1^{+} and PD-L1^{-} expression by TILs did not reveal statistically significant differences (DFS: p=0.456, one-, 3- and 5-year DFS rates: TILs-PD-L1^{++} 63%, 55%, 50%; TILs-PD-L1^{+} 78%, 54%, 42%; TILs-PD-L1^{-} 54%, 32%, 29%, respectively, Figure 3a; OS: p=0.531, one-, 3- and 5-year OS rates: TILs-PD-L1^{++} 88%, 50%, 45%; TILs-PD-L1^{+} 84%, 62%, 45%; TILs-PD-L1^{-} 59%, 40%, 37%, respectively; Figure 3b).

Association of clinicopathological parameters with OS and DFS as determined by univariate and multivariate cox regression analysis

By univariate analysis, tumor size, lymph node status, grading, tumor stage, PD-L1 expression by cancer cells, and PD-1 expression by cancer cells and TILs showed significant associations with OS and DFS (Table 3). No influence on OS and DFS was detected for PD-L1 expression by TILs (Table 3). The time period of resection as a potential impacting factor on OS or DFS was excluded by including it in univariate and multivariate analysis as a continuous and a grouped variable.

By multivariate analysis, high PD-L1 expression by cancer cells proved to be an independent prognosticator of both DFS and OS (DFS: Hazard ratio (HR): 0.76, 95%CI: 0.58-0.98, p=0.038; OS: HR: 0.75, 95%CI: 0.57-0.99, p=0.042, respectively, Table 3). Patients with positive lymph node status showed a significantly increased risk for disease progression (HR: 1.69, 95% CI 1.37-2.08, p<0.001) and death (HR: 1.66, 95% CI 1.34-2.06, p<0.0019) (Table 3). Tumor size was only found to significantly affect DFS (HR: 1.5, 95% CI 1.08-2.09, p=0.016), but not OS (p=0.077), by multivariate analysis (Table 3).

Discussion

In this study we evaluated the expression of PD-L1, PD-L2 and PD-1 and its potential clinical relevance in a comparatively large cohort of patients with esophageal and gastroesophageal junctional adenocarcinomas. Protein-expression of PD-L1, PD-L2 and PD-1 were quantitated for both cancer cells and TILs. [8,25,26] PD-L1 expression by esophageal cancer cells was found in 43.5% (73/168) of patients and PD-L1 expression by TILs was detected in 69% (116/168) of patients. These findings are in line with a recently published meta-analysis reporting PD-L1 positivity in around 50% of gastrointestinal tract cancers. [27] Increased expression of PD-L1 by cancer cells was found to be an independent factor of favorable outcome in our patient cohort, as determined by multivariate analysis. Additionally, 77.4% (130/168) of tumors were cancer cell-PD-1$^+$ and 81% (136/168) contained PD-1$^+$ TILs. We previously reported that expression of the PD-1 receptor by both cancer cells and TILs is associated with advanced tumor stage in esophageal adenocarcinoma. [28] Increased expression of PD-1 was found to correlate with higher tumor stage and lymph node involvement. Although it was not found to be an independent predictor of outcome, TIL-PD-1$^+$ and cancer cell-PD-1$^+$ patients demonstrated significantly reduced DFS rates, as determined by univariate analyses. Interestingly, cancer cell-PD-1$^+$ patients additionally showed decreased OS by univariate analysis. In the current study, we found that cancer cell-PD-L1$^+$ patients show both improved DFS and prolonged OS, determined by multivariate analysis. Consistent with these opposing findings of PD-1 vs. PD-L1 expression with DFS and OS, we further described in this study that a high number of cancer cell-PD-1$^-$ (38/168, 22.6%) and TILs-PD-1$^-$ (32/168, 19.1%) tumors demonstrated high expression of PD-L1 on both cancer cells (24/38, 63.2%) and TILs (24/32, 75%). The fact that high levels of an immune escape mechanism, PD-L1,

correlate with favorable clinical outcome is opposite of what one might have anticipated. One possible explanation for this finding could be that an inflamed tumor microenvironment containing high levels of T-effector cell infiltrates and inflammatory cytokines, including interferons (IFNs), could result in elevated expression levels of the IFN target gene, PD-L1. [8] [29] PD-L1 might be up regulated after induction of immune responses leading to proliferation of T-cells and subsequently secretion of anti-tumoral cytokines such as IL-10 or interferon-γ. [20] [30] Additionally, CD8+ T-cells and various other cytokines present in the tumor microenvironment (e.g. IL-2, interferon-γ, IL-7, IL-15 and IL-21) can promote the expression of PD-L1. [31] [32] [33] PD-L1 expression may thus not necessarily coincide with an immunosuppressed microenvironment per se, but rather serve as a proxy for immune activation. Therefore, increased PD-L1 expression might result from an adaptive immunological tumor-host relationship. [34] [8] Consistently, localized PD-L1 expression has been shown to advocate organ-specific autoimmunity. [35] On the other side, other receptors as for example B7-1 (CD80) have been described to interact with PD-L1 and might lead to bidirectional inhibitory responses in T cells. [36,37]

Consistent with this possibility and the findings reported herein, a correlation of PD-L1 expression with positive prognosis has recently also been described in a study involving immunohistochemical analysis of tissue microarrays of 177 patients with advanced esophageal squamous cell carcinoma (ESCC). [38] Additionally, Jesinghaus and colleagues recently reported a significant association of high PD-L1 expression by cancer cells with improved OS and DFS in patients with ESCC. [34] They evaluated PD-1 and PD-L1 expression immunohistochemically in 125 therapy-naïve ESCC patients and demonstrated a relationship between the presence of intraepithelial CD3+ TILs and high PD-L1 expression. [34] In a separate study [39] assessing PD-1 and PD-L1 expression in tumor specimens from 349 patients with ESCC, increased PD-L1

expression was similarly found to significantly correlate with favorable clinical outcome. [39] Although this stands in contrast to other studies reporting a less favorable outcome for PD-L1 positive ESCC, [18] [19] [20] [21] [22] a beneficial outcome of PD-L1 expression has also been described for various additional tumor entities, such as non-small cell lung cancer, colorectal carcinoma, melanoma, or Merkel cell carcinoma. [40] [41] [8] [42] Another study investigating PD-L1 pathway member expression in a tissue microarray of 464 patients with gastric adenocarcinoma found a significant correlation of both, PD-L1 and PD-1 with adverse prognostic pathological factors and OS. [43] The fact that in our cohort a PD-1^{+} PD-L1^{-} expression pattern is associated with an unfavorable patient prognosis, could potentially relate to the possibility that PD-1 receptor expression by cancer cells may promote cancer progression by triggering tumor cell-intrinsic growth signals, a mechanism previously reported in melanoma. [44]

Most studies evaluating the PD-1 pathway in esophageal carcinoma have been performed in patients with ESCC, whereas its role in esophageal adenocarcinoma is poorly understood. Derks and colleagues assessed PD-1 pathway member expression in tissue microarrays of 354 patients with esophageal adenocarcinoma. [45] This group reported an association of PD-L2 expression with Barretts's esophagus and furthermore described a potential association of the inflammatory environment in Barretts's esophagus and PD-1 ligand expression. [45] In our study, PD-L2 was only occasionally expressed on cancer cells or TILs (3.5% and 1.8%, respectively). Indeed, PD-L2 has mainly been reported in macrophages and antigen-presenting cells. Nevertheless, some solid cancers, such as hepatocellular and endometrial carcinoma, have also shown cancer cell expression of PD-L2. [46-48] The relative rarity of PD-L2 in our patient cohort makes it difficult to estimate its potential clinicopathological impact or tumor immuno-biological significance in esophageal adenocarcinoma. The fact that the immunohistochemistry for PD-L2 is not as well established when compared to

immunohistochemistry with PD-L1 might explain differences in studies and a limited significance of PD-L2 expression in patients with AEG.

To our knowledge, this represents one of the largest studies evaluating PD-1, PD-L1, and PD-L2 expression patterns in esophageal adenocarcinoma. Our study revealed, that expression of PD-L1 by cancer cells and by TILs is an independent predictor of improved DFS and OS in patients with esophageal adenocarcinoma. On the other side, PD-L2 expression was only rarely detected in our patient cohort. Additionally, we could describe that TIL-PD-1$^+$ and cancer cell-PD-1$^+$ patients demonstrated significantly reduced DFS rate in univariate analysis. Together, these findings highlight the potential importance of the PD-1 pathway in AEG development and underline the potential promise of PD-1 therapies for this devastating malignancy.

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Figure legends

Figure 1. This figure depicts representative images for (a) negative (0) PD-L1 staining on cancer cells, (b) 2+ and (c) 3+ positive PD-L1 staining on cancer cells as well as (d) negative (0) PD-L1 staining by TILs (e), 2+, and (f) 3+ positive PD-L1 staining by TILs.

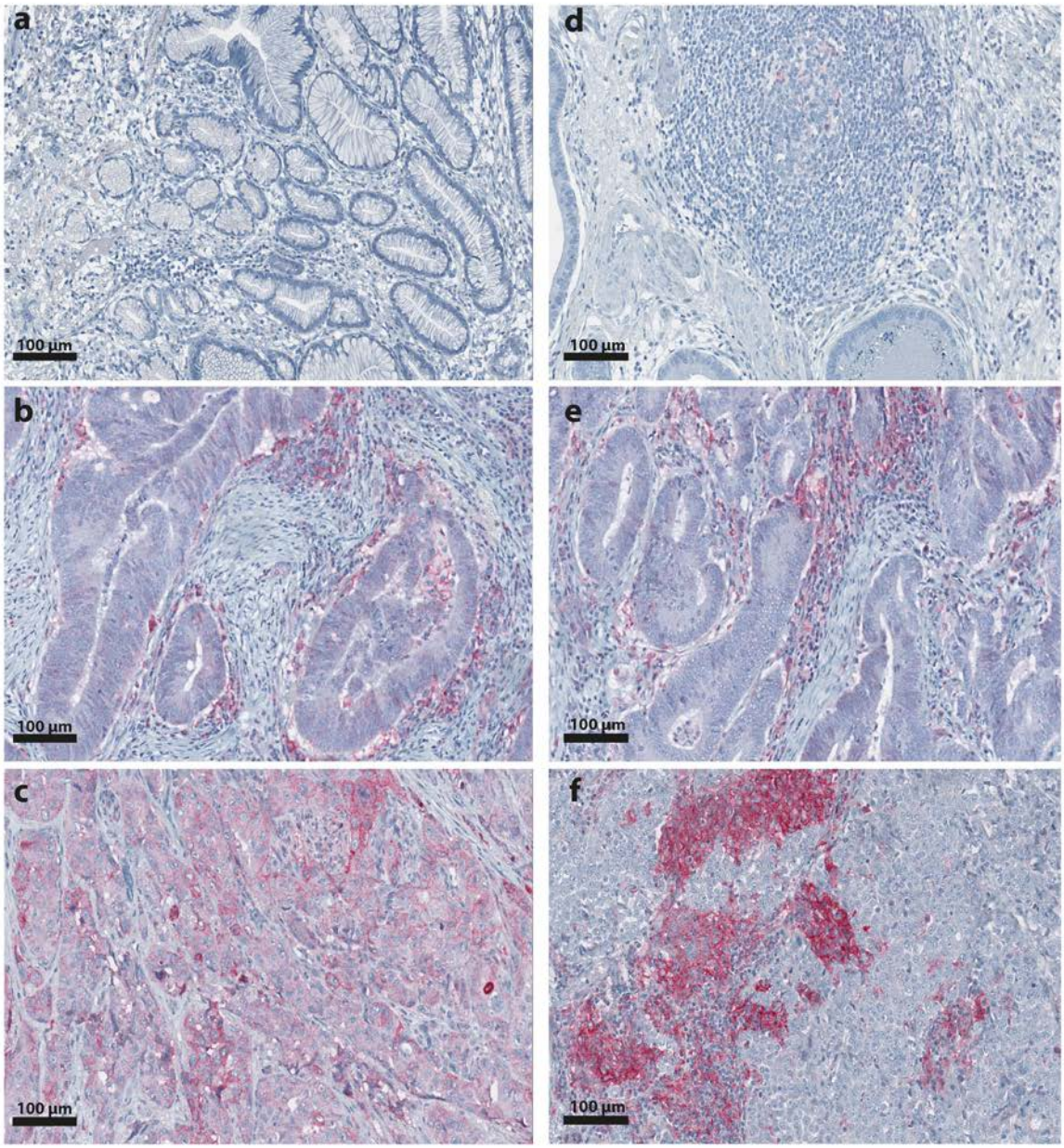
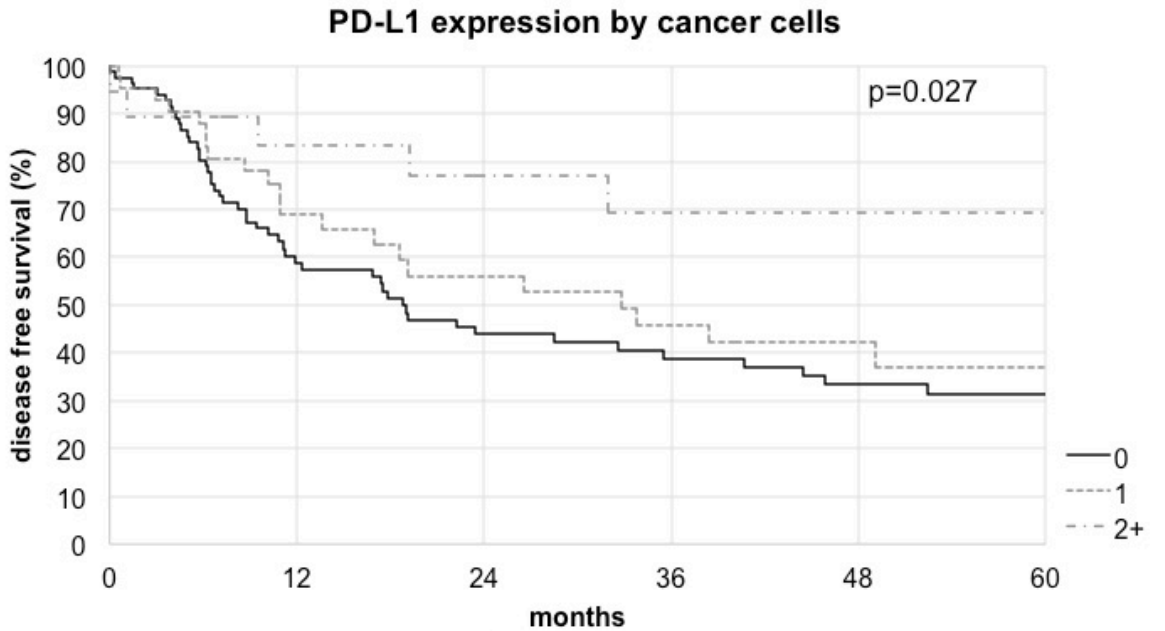
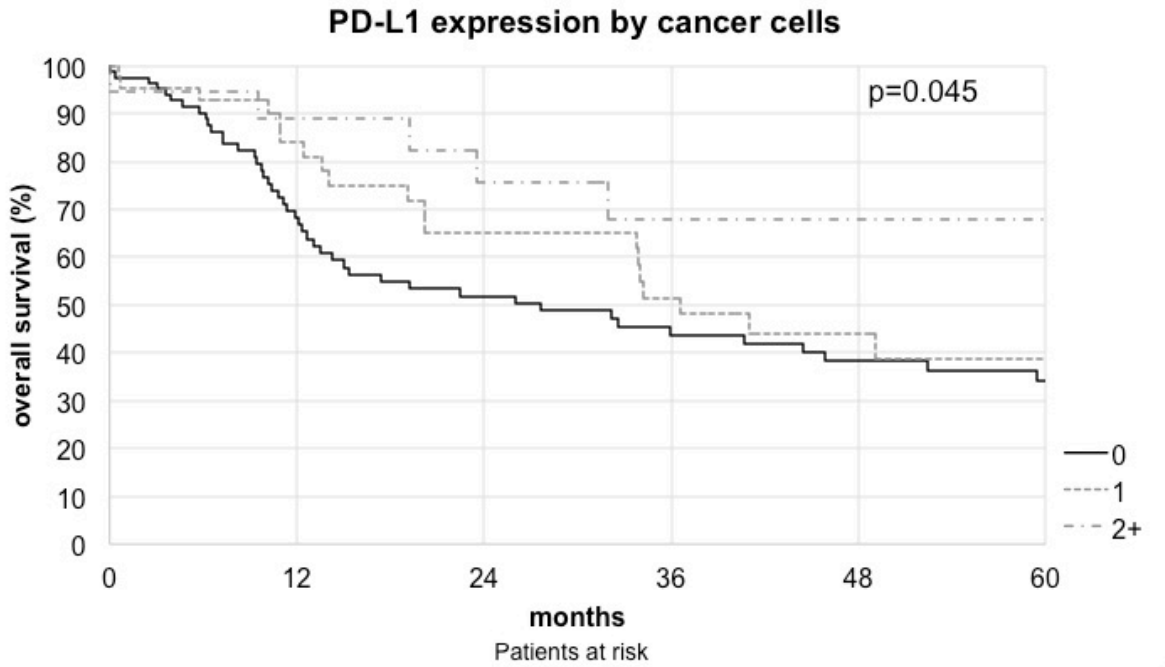

a
100 µm
d
100 µm
b
100 µm
e
100 µm
c
100 µm
f
100 µm

Figure 2. Influence of PD-L1 expression by cancer cells on a) Disease free survival (DFS) and b) overall survival (OS). Patients were grouped in three categories according to the PD-L1 expression levels by cancer cells: 0 (0%, black line), 1 (1-25%, dark grey dotted line) and 2+ (26-100%, light grey dotted line). Patients at risk for each category and time point are summarized in the table blow the graph.



Patients at risk

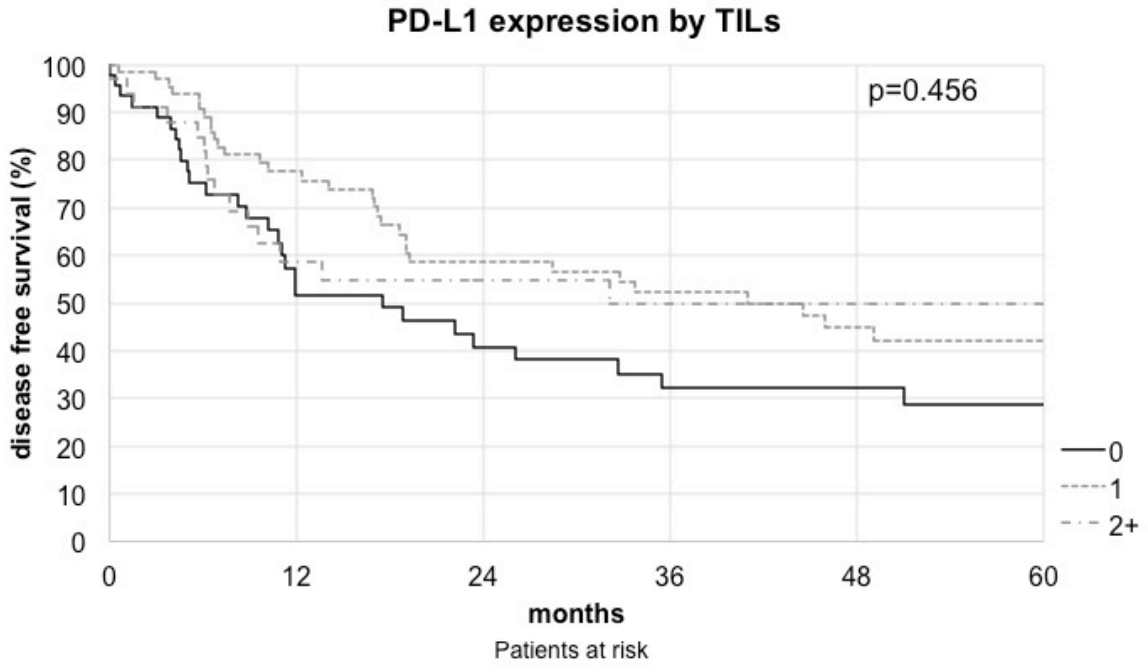
0	83	42	31	24	19	15
1	43	24	19	15	11	8
2+	19	16	12	11	11	11



Patients at risk

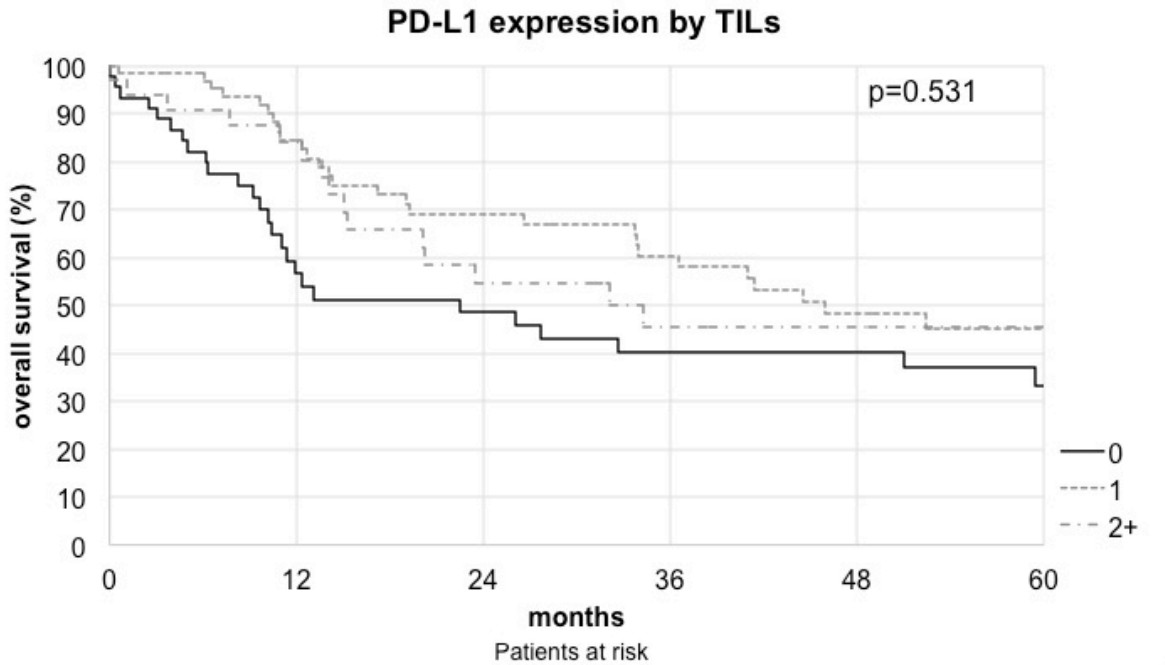
0	83	48	36	28	22	18
1	43	30	22	17	12	8
2+	19	17	13	11	11	11

Figure 3. Influence of PD-L1 expression by TILs on a) Disease free survival (DFS) and b) overall survival (OS) Patients were grouped in three categories according to the PD-L1 expression levels by TILs: 0 (0%, black line), 1 (1-25%, dark grey dotted line) and 2+ (26-100%, light grey dotted line). Patients at risk for each category and time point are summarized in the table blow the graph.



Patients at risk

0	46	21	17	12	11	8
1	66	44	31	26	19	16
2+	33	17	14	12	11	10



Patients at risk

0	46	23	20	15	14	11
1	66	47	35	29	20	16
2+	33	25	16	12	11	10

Table 1. PD-L1 expression by cancer cells in 168 patients with esophageal adenocarcinoma and its association with different clinicopathological findings

	Adenocarcinoma (n=168) n (%)	**PD-L1^{+} cancer cells (n= 73) n (%)**	**PD-L1^{-} cancer cells (n=95) n (%)**	**p-value**
Tumor size				
High-grade dysplasia	4 (2.4)	2 (2.7)	2 (2.1)	0.045
pT1a	13 (7.7)	8 (11)	5 (5.3)	
pT1b	20 (11.9)	13 (17.8)	7 (7.4)	
pT2	49 (29.2)	14 (19.2)	35 (36.8)	
pT3	77 (45.8)	33 (45.2)	44 (46.3)	
pT4	5 (3)	3 (4.1)	2 (2.1)	
Lymph node status				
pNx	13 (7.7)	8 (11)	5 (5.3)	0.520
pN0	61 (36.3)	29 (39.7)	32 (33.7)	
pN1	31 (18.5)	11 (15.1)	20 (21)	
pN2	26 (15.5)	10 (13.7)	16 (16.8)	
pN3	37 (22)	15 (20.5)	22 (23.2)	
Histologic grading				
G1	7 (4.2)	3 (4.1)	4 (4.2)	0.584
G2	74 (44)	29 (39.7)	45 (47.4)	
G3	87 (51.8)	41 (56.2)	46 (48.4)	
Neoadjuvant therapy				
No	105 (62.5)	47 (64.4)	58 (61)	0.748
Yes	63 (37.5)	26 (35.6)	37 (39)	
Total resection				
Yes	145 (86.3)	62 (84.9)	83 (87.4)	0.658
No	23 (13.7)	11 (15.1)	12 (12.6)	
Siewert classification				
AEG1	101 (60.1)	45 (61.6)	56 (58.9)	0.072
AEG2	44 (26.2)	14 (19.2)	30 (31.6)	
AEG3	23 (13.7)	14 (19.2)	9 (9.5)	
PD1 expression by tumor cells				
0 (0%)	38 (22.6)	24 (32.9)	14 (14.7)	0.016
1+ (1-25%)	36 (21.4)	11 (15.1)	25 (26.3)	
2+ (26-50%)	30 (17.9)	8 (11)	22 (23.2)	
3+ (51-75%)	43 (25.6)	20 (27.4)	23 (24.2)	
4+ (76-100%)	21 (12.5)	10 (13.7)	11 (11.6)	
PDL1 expression by tumor cells				
0 (0%)	95 (56.5)			
1+ (1-25%)	51 (30.4)			
2+ (26-50%)	12 (7.1)			
3+ (51-75%)	8 (4.8)			
4+ (76-100%)	2 (1.2)			

Table 2. PD-L1 expression by tumor infiltration lymphocytes (TILs) in 168 patients with esophageal adenocarcinoma and its association with different clinicopathological findings

	Adenocarcinoma (n=168) n (%)	**PD-L1^{+} TILs (n=116) n (%)**	**PD-L1^{-} TILs (n=52) n (%)**	**p-value**
Tumor size				
High-grade dysplasia	4 (2.4)	4 (3.4)	0	0.128
pT1a	13 (7.7)	8 (6.9)	5 (9.6)	
pT1b	20 (11.9)	17 (14.7)	3 (5.8)	
pT2	49 (29.2)	28 (24.1)	21 (40.4)t	
pT3	77 (45.8)	56 (48.3)	21 (40.4)	
pT4	5 (3)	3 (2.6)	2 (3.8)	
Lymph node status				
pNx	13 (7.7)	11 (9.5)	2 (3.8)	0.396
pN0	61 (36.3)	43 (37)	18 (34.6)	
pN1	31 (18.5)	22 (19)	9 (17.3)	
pN2	26 (15.5)	19 (16.4)	7 (13.5)	
pN3	37 (22)	21 (18.1)	16 (30.8)	
Histologic grading				
G1	7 (4.2)	5 (4.3)	2 (3.8)	1
G2	74 (44)	51 (44)	23 (44.2)	
G3	87 (51.8)	60 (51.7)	27 (51.9)	
Neoadjuvant therapy				
No	105 (62.5)	71 (61.2)	34 (65.4)	0.730
Yes	63 (37.5)	45 (38.8)	18 (34.6)	
Total resection				
Yes	145 (86.3)	99 (85.3)	46 (88.5)	0.809
No	23 (13.7)	17 (14.7)	6 (11.5)	
Siewert classification				
AEG1	101 (60.1)	73 (62.9)	28 (53.8)	0.252
AEG2	44 (26.2)	26 (22.4)	18 (34.6)	
AEG3	23 (13.7)	17 (14.7)	6 (11.5)	
PD1 expression by TILs				
0 (0%)	32 (19.1)	24 (20.7)	8 (15.4)	0.034
1+ (1-25%)	56 (33.3)	31 (26.7)	25 (48.1)	
2+ (26-50%)	68 (40.5)	50 (43.1)	18 (34.6)	
3+ (51-75%)	12 (7.1)	11 (9.5)	1 (1.9)	
4+ (76-100%)	0	0	0	
PDL1 expression by TILs				
0 (0%)	52 (31)			
1+ (1-25%)	77 (45.8)			
2+ (26-50%)	29 (17.3)			
3+ (51-75%)	10 (6)			
4+ (76-100%)	0			

Table 3. Influence of clinicopathological findings on overall survival (OS) and disease free survival (DFS) analysed by univariate and multivariate cox regression.
Footnote: CI (confidence interval), HR (Hazard ratio)

	univariate				**multivariate**		
	HR	**CI**	**p-value**		**HR**	**CI**	**p-value**
OS							
pT	1.85	1.40-2.45	<0.001		1.36	0.97-1.91	0.077
pN	1.79	1.48-2.16	<0.001		1.66	1.34-2.06	<0.001
Grading	1.83	1.23-2.72	0.003				
Tumor stage	1,33	1.20-1.48	<0.001				
PD-L1 expression by cancer cells	0.75	0.57-0.99	0.045		0.75	0.57-0.99	0.042
PD-L1 expression by TILs	0.91	0.69-1.21	0.531				
PD1 expression by cancer cells	1.28	1.09-1.51	0.003		1.09	0.86-1.39	0.484
PD1 expression by TILs	1.32	1.04-1.69	0.025		0.97	0.69-1.38	0.875
DFS							
pT	1.93	1.47-2.54	<0.001		1.50	1.08-2.09	0.016
pN	1.80	1.50-2.16	<0.001		1.69	1.37-2.08	<0.001
Grading	1.84	1.25-2.71	0.002				
Tumor stage	1,40	1.26-1.55	<0.001				
PD-L1 expression by cancer cells	0.74	0.56-0,97	0.027		0.76	0.58-0.98	0.038
PD-L1 expression by TILs	0.90	0.68-1.19	0.456				
PD1 expression by tumor cells	1.20	1.03-1.40	0.022		0.91	0.73-1.14	0.411
PD1 expression by TILs	1.36	1.07-1.72	0.012		1.14	0.82-1.60	0.440